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Evaluation of Renal Function in Neonatal Rats

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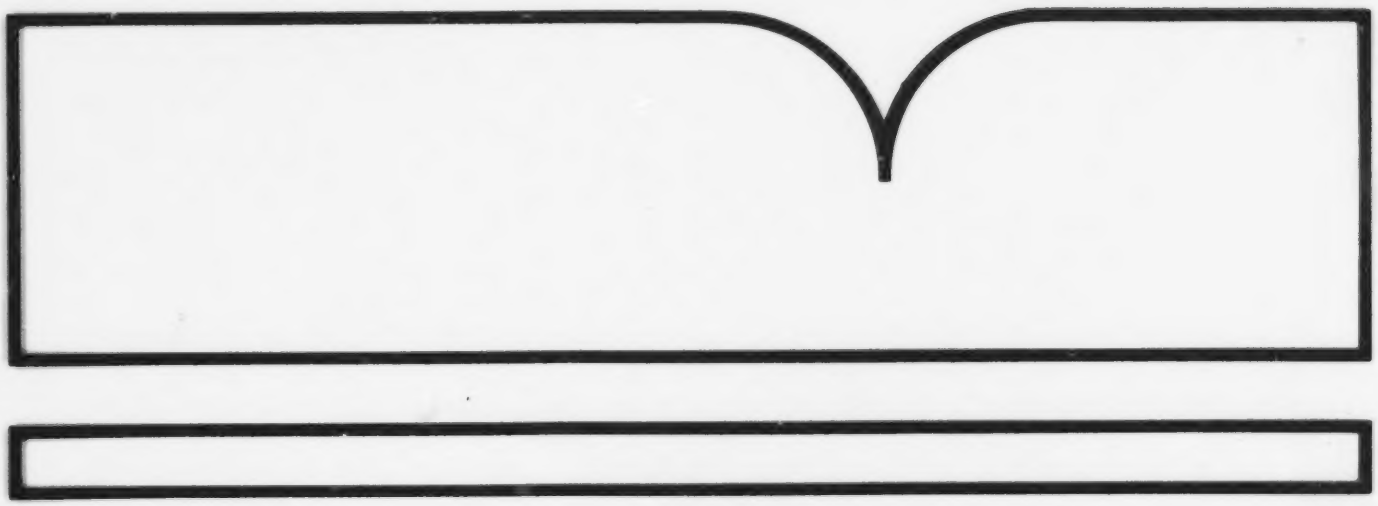

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16. ABSTRACT

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Evaluation of Renal Function in Neonatal Rats[1]

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Key Words. Renal function · Development · Neonatal rat · Hydropenia · Diuresis · Anti-diuresis · Nephrogenesis

Abstract. The ontogenetic profile of several parameters of neonatal renal development in the rat is presented. Nephrogenesis was observed to continue at a rapid pace between birth and 8 days of age and to be virtually complete by 11 days of age. The activity of alkaline phosphatase, a brush border enzyme, declined during this time period relative to organ growth as a whole. The ability to elaborate a concentrated urine when presented with a period of fluid deprivation was barely present at birth and increased dramatically with age. Finally, the diuresis response to an orally administered water load was detected on the second postnatal day, while the response to antidiuretic hormones was present to a slight degree on the first postnatal day.

In recent years, research in the area of developmental toxicology has been directed at examining the effects of perinatal exposure to chemicals on the physiology and behavior of the postnatal animal. This present research was undertaken to explore ways of evaluating renal function in the neonatal rat. The kidney provides an exceptionally well-suited system for such a study because its function is well understood in terms of morphology and because the urine, which represents the product of its physiological function, can be easily collected and analyzed using noninvasive techniques. Subsequent research reports will evaluate the sensitivity of the techniques described in this paper for the detection of perinatally induced renal dysfunction.

The development of the kidney in the neonatal rat has been described from the viewpoint of the glomerulus [3, 20], the renal tubules [4, 21], cytology [5], and biochemistry [10, 13, 33]. Studies on the development of renal functional capabilities in the neonatal rat began with the water loading experiments of *Heller* [14], *McCance and Wilkinson* [22], and *Falk* [9], and were quickly followed by investigations into the actions of various hormones [1, 7, 16, 19], the response to solute loading [11, 12, 18, 23, 31], the development of the renin-angiotensin system

[1] This paper has been reviewed by the Health Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

[34], and of the ontogeny of tubular transport [10, 29] and glomerular filtration [2, 6, 17, 25, 28]. Central to all these studies is the finding that pronounced changes in the morphology andfunctional capability of the rodent kidney occur during neonatal development. It was the intent of this study to refine the ontogenic profile of several basic renal functional and morphological parameters. The development of glomeruli was examined by evaluating the response to a water load and by quantitating their histological formation; the renal tubules by determination of the activity of alkaline phosphatase (an enzyme primarily located in the brush border of the tubules [32]); the loop of Henle by assessing the ability to concentrate urine in the absence of fluid intake; and the collecting ducts by assaying the response to antidiuretic hormones.

Methods

Animals

90-day-old, time pregnant, Sprague-Dawley rats were obtained from Charles River Breeding Laboratory, Wilmington, Mass. The day that sperm was found in the vaginal smear was considered day 1 of pregnancy. Animals were housed individually in plastic cages on pine shavings bedding in a bio-clean room. Food (Purina rat chow) and water were allowed ad libitum. Temperature was maintained at 20–24 °C, humidity at 40–50% and the diurnal cycle lasted from 6 a.m. to 6 p.m. In our laboratory, this strain normally delivers the evening of day 22 of gestation and the following day was considered day 1 of postnatal life (PD 1). On PD 1, all litters were reduced to a maximum of 10 pups with a sex ratio as equal as possible.

Collection of Urine

Micturition was induced by a technique similar to that described by *Falk* [9]. The pups were held ventral side upward and gently stroked in the perineal region with a microcapillary tube (Scientific Products). This action resulted in a reflex constriction of the bladder and expulsion of urine. Urine expressed by this technique could be quantitatively collected in the capillary tubes until pups reached around 12 days of age at which time the fur would trap some fluid. The tubes were capped (Capillary Tube Sealant, Scientific Products) and centrifuged briefly (Micro-hematocrit Centrifuge, Clay Adams) to remove air bubbles trapped during the collection process. The volume of fluid in the tubes was then determined by measuring to the nearest millimeter the distance from the plug to the meniscus. Capillary tubes were calibrated by pipetting known microliter quantities of distilled water into the tubes and measuring the length of fluid.

This technique took advantage of the fact that neonatal rats cannot micturate voluntarily, but require some external stimuli to void their bladders. Thus, by isolating pups from the dams, the exact quantity of urine produced in a given time period could be determined by first voiding the bladder, placing the pups in 250-ml Griffen beakers that contained bedding to a depth of about 5 cm, and at timed intervals voiding the bladders and collecting the urine. Because rat pups are poikilothermic during the ages studied [1], the beakers were placed in a water bath to a depth of 1–2 cm and the temperature at the surface of the bedding was maintained at 32–34 °C.

Hydropenia Response

On PD 1, 4, 5, 8, and 11 a male and female from each of five litters were removed from the nest, their bladders were then voided and a specimen of urine saved. The pups were then placed in the warmed beakers without access to fluids. 3 h after removal from the nest, the bladders were voided and the urine discarded. The initial 3-hour voiding period was incorporated into the protocol to ensure that the bladder did not become overdistended during the isolation period and that the urine used to determine the final osmotic pressure was elaborated only after the cessation of fluid intake was sensed by the pups. 6 h after removal from the nest, the bladders were again voided and a urine specimen saved to assess the ability to elaborate a concentrated urine. Additional male and female pups from each litter were treated similarly except they were returned to the nest each time. The osmotic pressure of an 8-µl aliquot of the urine specimens was then determined using a Wescor vapor pressure osmometer (Model 5001B, Wescor Inc., Logan, Utah). The pups that remained in the nest were used to ensure that no diurnal fluctuations in urinary osmotic pressure occurred.

Biochemistry and Morphology

At the conclusion of the above experiment, the hydropenic animals were decapitated and the kidneys were removed, weighed, and preserved for further study. The left kidney was fixed in a solution of 5% formaldehyde, 5% glacial acetic acid, and 70% ethanol, embedded in paraffin, and 8-μ cross-sections spaced approximately 60 μ apart (the diameter of glomeruli in rats of these ages [3]) were taken through the mid-hilar region. The sections were then stained with hematoxylin and eosin, and the number of fully formed glomeruli were counted. A qualitative index was used to describe the thickness of the nephrogenic zone in the renal cortex. The right kidney was frozen in a dry ice/acetone bath and stored in plastic vials at −60 °C. The kidney was subsequently thawed, homogenized in a 2-ml Potter-Elvehjem tissue homogenizer in pH 7.4 Hepes buffer (5% w/v homogenate), centrifuged at 5,000 *g* for 15 min in a table top centrifuge (Model CL, International Equipment Co.), and assayed for alkaline phosphatase activity using Sigma 104 phosphatase substrate (Sigma Chemical Co.) and protein by the Peterson modification of the Lowry technique [24]. Bovine serum albumin (Sigma Chemical Co.) was used as the protein standard. Absorbance readings were recorded at appropriate wavelengths using a Beckman Model 35 spectrophotometer. Alkaline phosphatase activity was expressed in Units (micromoles *p*-nitrophenol produced per hour).

Diuresis Response

On PD 1, 2, 3, 6, and 8, three males and three females from each of six litters were removed from the nest and their bladders were voided. Following weighing, each pup received an intragastric load of deionized distilled water equal to 5% of their body weight. Administration of the water load was achieved through use of microbore tubing (0.030 inch OD, Scientific Products) attached via a 30-gauge needle to a 500-μl Hamilton syringe. The tubing was passed down the esophagus to a point approximating the level of the stomach. In addition to receiving the water load, a pair of male and female littermates also received a subcutaneous injection of either antidiuretic hormone (lysine vasopressin, LVP; Sigma Chemical Co.) or an analogue of LVP [30] that lacks pressor activity (desmopressin acetate, DDAVP; Ferring Pharmaceuticals, Inc.) at a dosage fo 100 mU/kg. After administration of the water load or water load plus hormone, all pups were placed in warmed beakers. Every 30 min for a 150-min period, the pups were removed from the beakers and the urine produced during the time period was collected and quantified. Results are expressed as the percent of the water load excreted per interval for pups receiving only water and as the difference between the percent excreted by the water-loaded pups and the percent excreted by the pups who also received a hormone.

Statistical Analyses

The data were analyzed using analysis of variance techniques available in the Statistical Analysis System (SAS) [27]. Sex and age were used as main effects in the models. When a significant probability of a main effect was detected, the means were compared using Duncan's Multiple Range Test.

Results

Hydropenia

The ability of neonatal rat pups to concentrate their urine in response to 6 h of fluid deprivation is depicted in figure 1. Basal urinary osmolality (that recorded upon the initial removal of a pup from the nest) increased significantly between PD 1 and 4 (from 274 to 442 mOsm/kg) and by PD 11 reached 480 mOsm/kg. For those pups that remained in the nest during the 6-hour determination period, the final urinary osmolality was significantly affected by age, but there was no difference between the final and the basal osmolality for these pups. Therefore, there are no diurnal fluctuations in urine osmolality for pups remaining in the nest. The final osmolality of the urine for those pups that were removed from the nest was also significantly affected by age. However, unlike that observed for the non-fluid deprived pups, there was a pronounced age effect ($p < 0.001$) on the difference between the final and basal osmotic pressures. The final urine osmolality was 76 mOsm/kg above basal level on PD 1, increased to 152 mOsm/kg above basal on

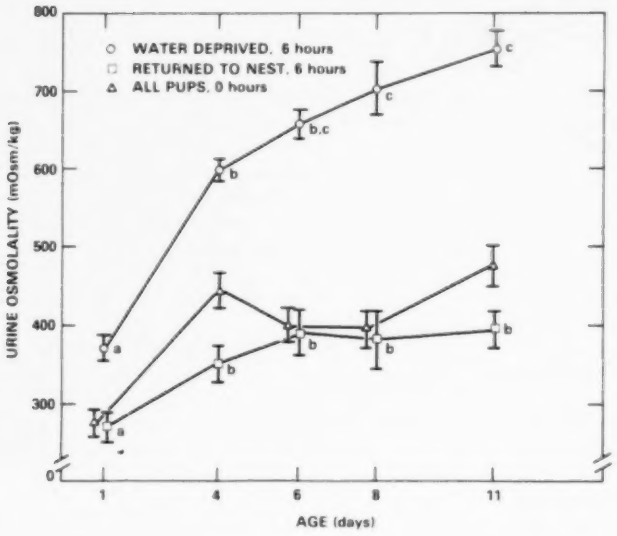


Fig. 1. Development of the hydropenic response in neonatal rats. Data are from a male and female pup from each of six litters on each day. No litter was retested. The vertical bars represent 1 SE. Points on the same line with the same superscript are not significantly different ($p > 0.05$) from one another (Duncan's Multiple Range Test). There is no difference between the initial urine osmolality and that obtained from the pups that remained in the nest for the duration of the 6-hour test.

Table I. Summary of the biochemical and morphological development of neonatal rat kidneys

	Age (postnatal days)		
	1	4	6
Number tested	12	12	12
Body weight, g	6.8 ± 0.1^{a}	10.4 ± 0.3^{b}	14.2 ± 0.5^{c}
Kidney weight			
Total, mg	36.7 ± 1.1^{a}	68.0 ± 1.8^{b}	102.9 ± 4.1^{c}
Relative, mg/g body weight	5.4 ± 0.1^{a}	6.6 ± 0.1^{b}	7.3 ± 0.2^{c}
Renal protein content			
Total, μg	$1,625 \pm 99^{a}$	$3,102 \pm 289^{a}$	$5,867 \pm 777^{b}$
Relative, μg/mg kidney	44 ± 2^{a}	$46 \pm 4^{a,b}$	61 ± 9^{c}
Renal alkaline phosphatase activity			
Total, units	126 ± 6^{a}	210 ± 13^{b}	279 ± 11^{c}
Relative, units/mg kidney	3.4 ± 4.1^{a}	$3.1 \pm 0.2^{a,b}$	$2.7 \pm 0.1^{b,c}$
Specific, Units/mg protein	80 ± 5^{a}	71 ± 4^{a}	$65 \pm 16^{a,b}$
Glomerular counts			
Total, number/cross-section	69 ± 4^{a}	110 ± 5^{b}	150 ± 8^{c}
Relative, number cross-section/mg kidney	1.9 ± 0.1^{a}	1.6 ± 0.1^{b}	1.5 ± 0.1^{b}
Nephrogenic zone, score	3.4 ± 0.2^{a}	3.2 ± 0.1^{a}	2.8 ± 0.1^{b}

Pups are from the hydropenia experiments. Data are presented as the mean ± SE. Means with the same superscript are not significantly different from one another.

PD 4, and reached 302 mOsm/kg above basal on PD 11. This physiologic response, which was barely significant on PD 1 ($p < 0.05$) increased dramatically by day 6 ($p < 0.001$) and leveled off between 8 and 11 days of age.

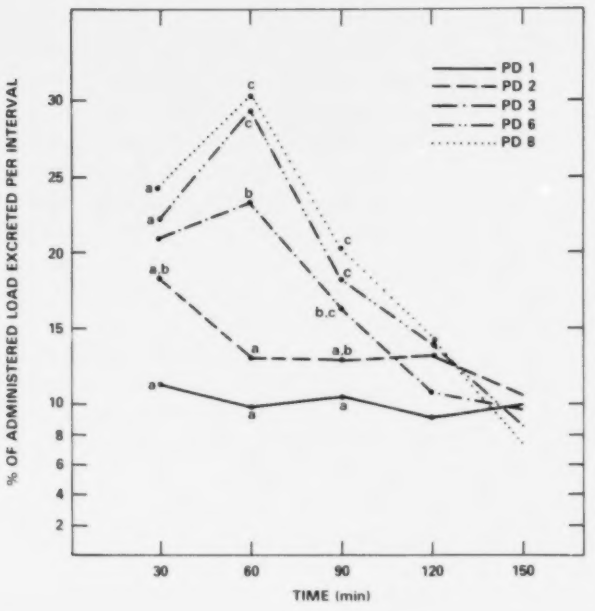
Biochemistry and Morphology

The developmental patterns for kidney weight, renal protein content, renal alkaline phosphatase, activity, and glomerulogenesis are presented in table I. The kidneys grew in a linear fashion between PD 1 and 11, increasing in weight by 382% during this time period. Kidney growth exceeded that of the body by 35% from PD 1 to 6, reaching a peak of 7.3 mg kidney/g body weight, and then equaled body growth through PD 11. Total renal protein also increased linearly with age, reaching a level by PD 11 654% above that present on PD 1. After adjusting for renal growth, the relative protein content (μg protein/mg kidney) increased 38% between PD 1 and 6 and remained approximately 60 μg/mg through PD 11. Total renal alkaline phosphatase activity increased by 258% between PD 1 and 11, but this increase was less than renal growth as a whole, as the relative activity (Units/mg kidney) declined by 27% between PD 1 and 8. Similarly, the specific activity of alkaline phosphatase declined during the neonatal period, leveling off by PD 8 at about 44% of the activity present at birth. The number of glomeruli present in midhilar cross-sections increased by 285% between PD1 and 8 and by an additional 10% by PD 11. The nephrogenic zone, which was very prominent on PD 1 and 4, declined through PD 8 and was no longer present by PD 11, indicating that the formation of glomeruli is nearly complete by this age.

		Statistical analyses (probability of main effect)	
8 11		sex	age
12 17.6 ± 0.6^{d}	12 25.4 ± 0.7^{e}	0.0157	0.0001
125.6 ± 3.1^{d} 7.1 ± 0.2^{c}	176.9 ± 6.8^{e} $7.0 \pm 0.2^{b,c}$	0.3393 0.1691	0.0001 0.0001
$7,396 \pm 411^{b}$ $58 \pm 3^{a,b}$	$10,638 \pm 802^{c}$ $59 \pm 3^{b,c}$	0.6393 0.9610	0.0001 0.0356
315 ± 16^{c} 2.5 ± 0.1^{c} 45 ± 4^{b}	451 ± 32^{d} 2.5 ± 0.1^{c} 43 ± 2^{b}	0.0866 0.0443 0.2417	0.0001 0.0001 0.0032
197 ± 10^{d} 1.5 ± 0.1^{b} 1.5 ± 0.2^{c}	216 ± 7^{d} 1.2 ± 0.1^{c} 0 ± 0^{d}	0.5968 0.9555 0.1626	0.0001 0.0001 0.0001

Water Diuresis

The developmental pattern for the renal response to an intragastric load of distilled water equal to 5% of the body weight is depicted in figure 2. Significant age effects on interval excretion patterns were noted in each of the first three observation periods ($p < 0.05$, 0.001 and 0.001 for the 30, 60, and 90 min periods, respectively), but not in the last two periods. For the most mature pups (PD 6 and 8), peak urine flows reached approximately 30% of the administered dose between 30 and 60 min, while during this same period the immature pups (PD 1 and 2) excreted less than 14% of the dose. Flow rates did not change with time in the PD 1 pups, remaining at 10–11% of the dose per interval. For 2-day-old pups, the peak flow



ANOVA p Value	Sex	0.5305	0.1334	0.3719	0.3686	0.5441
	Age	0.0384	0.0001	0.0005	0.0584	0.1602

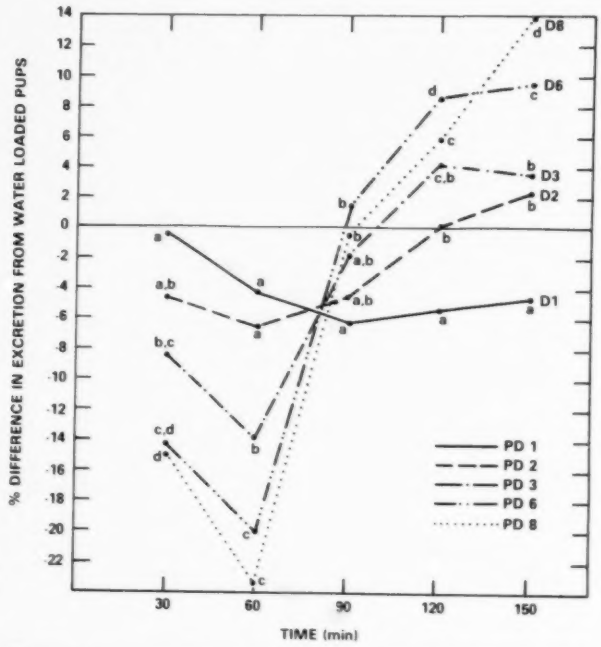
Fig. 2. Development of diuresis in response to a water load equal to 5% of the body weight. Data are from a male and female pup from each of six litters on each day. No pup was retested at a later age. Data presented as the percent of the administered dose excreted during each of the five observation periods. The statistical probability of the effect of sex and age as determined by Anova is presented for each time period below the graph. When a significant effect of age was detected, means were compared using Duncan's Multiple Range Test. Points in a vertical line with the same superscript are not significantly different from one another ($p > 0.05$).

occurred within 30 min of dosing, thus showing a temporal pattern different than observed in older pups. The half-life of the administered load was 150 min on PD 1, 105 min on PD 2, 70 min on PD 3, 60 min on PD 6, and 55 min on PD 8.

Hormone Response

The effects of co-administration of LVP or DDAVP and the water load are represented in figures 3 and 4. For simplicity of presentation, only the difference in percent excretion between the amount of urine excreted in a given interval by the animals receiving both the water load and the hormone and that by those receiving only the water load is depicted. The effect of the hormone can then be determined by testing whether this value is different from zero.

The ontogenic profile of LVP response indicates several important trends. There were age effects noted for every observation period except the third. Administration of LVP had no effects on urine production during the first observation period on PD 1, the third period on PD 3, 6, and 8 and during the fourth period on PD 2. Peak depression of urine formation was observed in the second observation period and amounted to approximately a 20% reduction relative to water-loaded pups on PD 6 and 8. Pups from PD 1 and 2 produced approximately 5% less in that period, while PD 3 animals were



ANOVA p Value						
	Sex	0.3796	0.5395	0.1987	0.7048	0.1891
	Age	0.0001	0.0001	0.0578	0.0001	0.0001

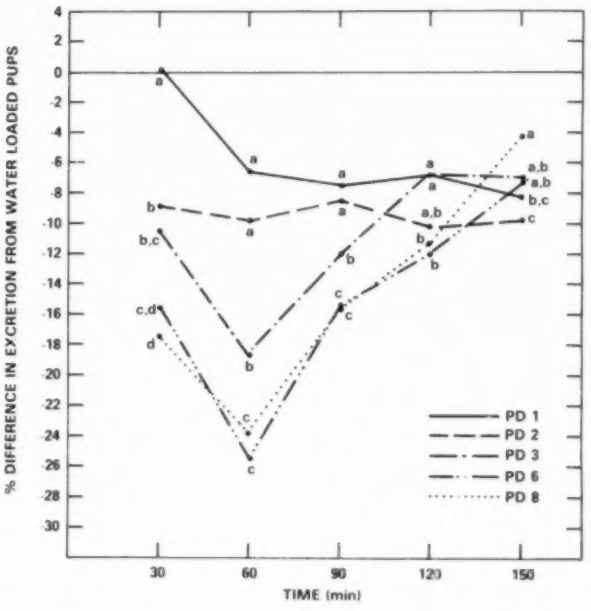
Fig. 3. Development of the antidiuretic response to LVP. Data are from a male and female pup from each of five litters. No pup was retested at a later age. Pups received the water load as described in figure 2 and in addition received a subcutaneous injection of 100 mU/kg lysine-vasopressin. Data presented as the *difference* in excretion per interval between the water-loaded pups and the hormone-administered pups. Statistical analysis as described in figure 2.

intermediate in response, producing 14% less.

During the first two observation periods, the profile of response to DDAVP closely resembled that of LVP at all ages. However, at later time periods, when the activity of LVP was waning, DDAVP continued to exert an effect on urine production. The first observation period on PD 1 was the only instance where DDAVP did not significantly reduce urine production relative to control level. The greatest depression of urine flow rates was observed in the two oldest age classes 60 min after water loading. On PD 8, flows were reduced 24% by LVP and 26% by DDAVP during that interval, indicating equal potency of both hormones.

Discussion

Relatively little quantitative data exists on either basal or hydropenic urinary osmotic pressures in neonatal rats. The data reported here for basal urinary osmotic pressures are lower than those reported by both *Falk* [9] and *Oh* et al. [23]. The former examined pups on PD 1 and PD 12–14 and reported pressures of 600 and 760 mOsm/kg, respectively, while the latter examined pups daily between PD 1 and 8 and saw a gradual rise from 373 mOsm/kg on PD 1 to 850 mOsm/kg on PD 7. For the hydropenic response, *Heller* [15] reported that newborn rats excreted a urine hypotonic to serum even after 24 h of fluid deprivation, while *Falk* [9] observed a



ANOVA p Value	Sex	0.8205	0.3116	0.7145	0.6083	0.9553
	Age	0.0001	0.0001	0.0001	0.0065	0.0022

Fig. 4. Development of the antidiuretic response to DDAVP. Methods similar to that described in figure 3 with the exception that the hormone is a vasopressin analog with relatively little pressor activity and a prolonged plasma half-life. Statistical analysis as described in figure 2.

rise in urine osmolality of 35% (270 mOsm/kg) in 12-day-old pups. All the previous studies required either the estimation of the osmotic concentration by refractometry or the pooling of the samples or addition of distilled water to make the volumes large enough for measurement. Using direct measurement of the urinary osmotic pressure, we have demonstrated that newborn pups can increase the concentration of their urine in response to fluid deprivation, and that the response further develops during the first postnatal week.

The data for glomerular development and alkaline phosphatase activity confirm previously observed trends in renal development. *Wacker* et al. [33] reported a decline in the specific activity of renal alkaline phosphatase activity between PD 1 and 14 followed by a rise to adult levels by PD 56. Activity on PD 1 was only 21% of the adult level. Both *Kettleson* [20] and *Arataki* [3] reported that glomerulogenesis was largely completed by 3 weeks of age, with the number of glomeruli more than doubling in this time. It is interesting to note that the development of glomeruli occurs in utero in humans, being largely complete by the 36th week of gestation [26]. As such, it represents a developmental activity that is largely missed by toxicology studies that use as a model the late-term rodent fetus.

The ability to excrete a water load during neonatal development has probably received

more attention than any other developing renal function. *McCance and Wilkinson* [22] observed no diuresis in newborn rats, as did *Heller* [16], *Falk* [9] and *Adolf* [1], but there is no agreement among these authors as to when the response first appears and when it resembles the adult response. Our results most closely resemble those of *Falk* [9], although he reported that maximum urine flows occurred 2–3 h after loading, whereas we observed maximum flows within 30–60 min. It should be noted that while water absorption in the alimentary tract is lower in newborn as opposed to adult rats [14], it is not of significant magnitude to account for the differences in water excretion in the age class examined [9].

The developmental response to antidiuretic activity has also been widely studied [7, 9, 16]. Our data compares most closely with that of *Falk* [9], but indicate a response to antidiuretic hormones at a younger age than previously reported. The difficulty in detecting a response to these hormones on PD 1 might be due to the fact that in all the studies the response is elicited following a water load. As pups at this age do not respond to a water load by increasing urine flow rates, the ADH response might appear differently than at older ages when that response is present. On PD 1, the pups are apparently refractory to the effect of the hormone during the first time period and show only mild responsiveness at later periods. It is of interest to note that as the pups matured the duration of the antidiuretic activity of LVP decreased appreciably. The half-life of LVP in adult rats is approximately 15 min. Thus, the increased urine flow rates in older LVP-treated animals in the last three periods might represent metabolic degradation of the hormone that was not possible in the more immature animals. The prolonged effect of DDAVP at all ages probably reflects the greater half-life of this analog compared to LVP [8]. It should also be noted that DDAVP has relatively little pressor activity [30], and therefore the effects on the kidney can be attributed to direct action on the collecting ducts and not to changes in glomerular perfusion pressures.

In summary, we have presented the ontogenetic profile of several renal developmental parameters in greater detail than has been presented earlier. Although differing in some details from earlier reports, we feel the application of a number of more modern techniques in our study provide our data with a firmer foundation. Several of the responses we observed were recorded at earlier ages than previously reported. The techniques are relatively simple to apply and require little or no equipment. As such, they may be readily applied to studies attempting to evaluate the presence of perinatally induced renal dysfunction.

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